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PROGRESS REPORT FOR NGR-48-001-004 for JANUARY TO JUNE, 1966

WORK COMPLETED

Work in our laboratory with Hydrogenomonas facilis established that glutamate is a product of short-term $C^{14}O_2$ fixation coupled with ribose oxidation, yet intermediates of the tricarboxylic acid (TCA) cycle were not labeled (1). Our data also indicated that function of the reductive pentose phosphate pathway (Calvin cycle)of CO_2 fixation was suppressed under these conditions. Because glutamate and 3-phosphoglycerate (PGA) were among the early identifiable products, their labeling patterns have been studied with the hope that these would provide clues about the mechanism of $C^{14}O_2$ incorporation.

Results are shown in Table 1.

Table 1. Labeling of Glutamate and PGA by $HC^{14}O_3^-$ during Ribose Oxidation by H. facilis

Fixation time	Per cent of C^{14} present in concentrated ethanol extract as		Per cent distribution by carbon					
			Glutamate			PGA		
	Glutamate	PGA	C_1	C_2-C_4	C_5	C_1	C_2	C_3
6-sec	3.9% (5.95 x 10^4)*	5.6% (8.49 x 10^4)	90	-----	10	101**	0	0
12-sec	3.8% (1.19 x 10^5)	9.7% (3.02 x 10^5)	48	-----	51	101**	0	1

*Bracketed numbers represent C^{14} found (expressed as d.p.m.).
**Corrected for incomplete (87%) absorption of CO_2 by hyamine hydroxide.

As is evident, label is incorporated exclusively into C_1 of PGA. In another 10-second fixation study under identical conditions radioisotope has been found exclusively in C_1 of pyruvate which was isolated as the 2,4-dinitrophenylhydrazone. In these short-term fixation studies our earlier observation that TCA-cycle intermediates were not labeled by $C^{14}O_2$ was confirmed. Thus it seems unlikely that the observed labeling of C_1 occurred via carboxylation, equilibration of the product with fumarate and subsequent decarboxylation. The data instead support the operation of the Calvin cycle, although at a suppressed rate, during

CO_2 fixation by H. facilis in the presence of ribose. The primary product, PGA, is apparently readily convertible to pyruvate. A less likely alternative pathway that cannot be ruled out at present would involve catalysis by pyruvate synthase resulting in reductive carboxylation of acetyl CoA. In this case, pyruvate, the primary product, would be converted to PGA.

It is also evident from Table 1 that glutamate is a minor early product of $C^{14}O_2$ fixation coupled with ribose oxidation. However, its labeling pattern is of interest, particularly in the absence of labeling of intermediates of the TCA cycle. As can be seen, C^{14} is first incorporated into C_1 and distributed then into C_5. Separate 10-second fixation studies have confirmed this in that 80% of the label was in C_1 and 20% in C_5. The data are consistent with the function of α-ketoglutarate synthase, which is known to catalyze a reductive carboxylation of succinyl CoA in photosynthetic bacteria. The product, α-ketoglutarate, is one of two intermediates of the TCA cycle whose labeling has not been investigated in H. facilis because of its instability. Distribution of radioisotope with time into C_5 of glutamate could reflect an independently operational pathway to glutamate or consecutive catalysis by α-ketoglutarate synthase, isocitrate dehydrogenase and isocitrate lyase followed by reversal of the latter two steps. Cleavage and resynthesis of isocitrate by isocitrate lyase would result in a redistribution of C^{14} in the isocitrate skeleton that could account for label incorporation into C_5 of glutamate. Indeed isocitrate lyase is readily detectable in ribose-grown cells (Table 2).

Failure to find C^{14} in intermediates of the TCA cycle was unexpected and might have been due to at least two conditions:

a) ribose-grown H. facilis does not have a functionally complete TCA cycle; b) ribose-grown H. facilis does not have the usual carboxylation reactions that afford continued regeneration of oxaloacetate from three-carbon precursors. Either of these conditions would have profound consequences in terms of cellular metabolism. Therefore assays for enzymes of the TCA cycle and for enzymes catalyzing major ancillary reactions were conducted. In general, assays were performed on cells grown on a ribose-minerals medium and on the rich medium, yeast extract, as a control. Results are shown in Table 2.

Table 2

Enzymes of the TCA Cycle and Ancillary Steps

Enzyme	Sp.Act. (μmoles/min/mg protein) x 10^3 in extracts (from cells cultured)					
	on ribose		on yeast extract		autotrophically	
	S_{3500}*	$S_{105,000}$	S_{3500}	$S_{105,000}$	S_{3500}	$S_{105,000}$
Citrate condensing enzyme	226	533	323	402	-	-
Aconitase	30	57	31	117	-	-
Isocitrate dehydrogenase (TPN^+)	148	569	71	309	-	-
Succinate dehydrogenase	323	0	610	883	-	-
Fumarase	341	1330	243	881	-	-
Malate dehydrogenase (DPN^+)	4360	12730	3530	9240	-	-
Malate dehydrogenase (TPN^+)	72	234	206	514	-	-
DPNH oxidase	21	22	52	19	30	-
Isocitrate lyase	-	16	-	23	-	22 (300)**
Malate synthase	-	-	-	65	-	27 (120)**

*Supernatant from centrifugation at 3500 g. Analogous symbology is used for other column headings.

**Bracketed values are for corresponding fractions from acetate-grown cells.

It is evident that most enzymes of the TCA cycle are detectable and are probably "constitutive" in H. facilis.

Several assays for pyruvate and α-ketoglutarate dehydrogenase have been conducted and results were uniformly negative (Table 3).

Table 3

Enzymes of the TCA Cycle and Ancillary Steps

"Absent" in both ribose- and yeast-extract grown cells:

Lactate dehydrogenase (DPN^+ or TPN^+)
Pyruvate dehydrogenase (DPN^+)
α-Ketoglutarate dehydrogenase (DPN^+)
Pyruvate carboxylase (ATP; cat. am'ts: biotin, acetylCoA)
Malic dehydrogenase, decarboxylating or "malic enzyme" (DPN^+ or TPN^+)
PEP carboxykinase (GDP or IDP)
PEP carboxylase

Catalysis by these enzymes requires at least two proteins and it seems likely that the integrity of the protein complex is lost during cell disruption. It is well known that both activities are difficult to detect in bacteria. Therefore, at present, little significance is given to the apparent absence of these activities in H. facilis. On the other hand, detection of the usual ancillary carboxylases is considerably easier and we therefore regard the absence of pyruvate carboxylase, "malic enzyme", phosphoenolpyruvate (PEP) carboxykinase and PEP carboxylase as quite significant. This would be consistent with glutamate labeling by $C^{14}O_2$ without detectable labeling of intermediates of the TCA cycle.

(1) Characteristics and Intermediates of Short-Term $C^{14}O_2$ Incorporation During Ribose Oxidation by Hydrogenomonas facilis, B. A. McFadden and H. R. Homann, J. Bacteriol., 89, 3 (1965).

WORK IN PROGRESS

As mentioned in the previous section, work with whole cells has suggested that the Calvin cycle functions at a diminished rate during heterotrophic CO_2 fixation. Work is in progress to identify the metabolic locus or loci that are sensitive to repression or feedback inhibition during heterotrophic culture. Assays of ribulose 5-phosphate kinase and ribulose diphosphate (RuDP) carboxylase in H. facilis cultured autotrophically and on various organic substrates are being completed. Catalysis by each of these enzymes is required only during autotrophic growth. It is

possible that the synthesis of these enzymes is coordinately regulated with that of hydrogenase.

One possible site of negative feedback control in the Calvin cycle is the step catalyzed by RuDP carboxylase. This enzyme is being purified from fructose-grown H. facilis in which its specific activity is comparable to that for the enzyme after autotrophic culture. After purification a number of properties of RuDP carboxylase will be examined including its interaction with plausible feedback inhibitors.

PROJECTED WORK

An assay of other tissues for the carboxylative enzymes apparently absent from H. facilis will be conducted. These experiments will serve as controls in the sense that our methods must detect these enzymes in tissues where they are known to be present. Assays will also be conducted for pyruvate and α-ketoglutarate synthases using Clostridial ferredoxin. The possibility of the presence of a comparable low-potential electron carrier in H. facilis will also be carefully considered.

Phosphoglycerate kinase will also be purified from H. facilis after establishing optimal conditions for its production. It seems quite likely that it catalyzes a step in the Calvin cycle that is subject to feedback regulation by adenylate.

After purification of RuDP carboxylase from fructose-grown H. facilis, its properties will be compared with the enzyme produced by a number of autotrophic species.